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INTRODUCTION:

Alcohol use impairs renal fluid handling and the ability to maintain adequate hydration. Of importance from a military readiness aspect, is that alcohol exposure causes physiological changes in fluid and electrolyte balance that will affect soldier performance. The soldier who uses alcohol is even more susceptible to dehydration especially when water is scarce, and from a pharmacological perspective, would be more susceptible to exposure to chemical warfare agents that would reach toxic levels in the dehydrated alcohol user faster than an individual with adequate hydration. A better understanding of renal fluid and electrolyte handling in response to alcohol is needed to design better treatments in dealing with fluid and electrolyte imbalances seen in alcoholics. It is difficult to interpret the mechanisms behind altered fluid handling associated with alcoholism because fluid and electrolyte balance appears to be affected differently at different stages of alcohol use. In this study, the role of vasopressin, an important hormone in body fluid regulation, in the physiological response to alcohol is being examined. In rat models of acute and chronic alcohol exposure, we are taking a systematic approach at elucidating the relationship between vasopressin synthesis in the brain, receptor regulation in the kidneys, and water and salt handling during different phases of alcohol exposure. The results of this research will lead to better strategies for management of fluid and electrolyte imbalance associated with alcohol use and will benefit military operational readiness by helping to provide medical countermeasures for soldiers who use alcohol.

PROGRESS:

Good progress has been made during the first year of this project as animal models of different phases of alcohol exposure have been developed, and whole animal renal function experiments have been successfully executed. The major specific tasks of 1) evaluating fluid and electrolyte regulating ability in models of acute and chronic alcohol exposure and 2) searching for mechanisms of altered fluid handling via *in vitro* assessment of vasopressin levels in the brain, blood and urine, are solidly underway. Research accomplishments associated with each task outlined in the Statement of Work are as follows:

1. *Fluid and electrolyte regulating ability experiments*

Animal models: We have established dependable animal models of acute and chronic alcohol exposure. The models developed can be studied repeatedly in several experiments, resulting in a considerable reduction of the total numbers of animals used in this study (about 25% of the original predicted numbers). With chronically implanted vascular and intragastric catheters and a bladder cannula staying patent and infection-free for weeks, we were able to run a baseline assessment of renal clearance, an experiment examining water load excretion, and a V2 antagonist dose response experiment all in the same animal. In some animals, an additional experiment examining maximum urine concentrating ability in response to the V2 agonist dDAVP was also done. Additionally, tissues were harvested from the same animals, for *in vitro* analyses of vasopressin and vasopressin receptor levels.

These models for acute alcohol exposure (up to 7 days of 15% ethanol, at a volume of 2% body weight, administered directly into the stomach via the indwelling i.g. line) and chronic alcohol exposure (8 weeks of 15% ethanol, 2% body weight volume per day in a liquid diet) provide exact and consistent delivery of alcohol that is non-stressful and well-tolerated by the animals. Blood alcohol concentrations reach about 100 mg/dL within 60 minutes of dosing. Experiments are run 18 hours after dosing for the acute alcohol exposure group and 18 hours after alcohol is withheld from the liquid diet for the chronic alcohol exposure group. On the day of the experiment blood alcohol levels had returned to undetectable levels. Thus, we were able to assess changes in physiological status resulting from alcohol use rather than the acute pharmacological effects of alcohol. There were no differences in baseline body weight, plasma osmolality or hematocrit between control and ethanol groups.

The added advantage of running several experiments in the same animal allows for control of variability between individual animals, and enables the generation of data with greater precision and detection of finer differences in physiologic regulation of fluid balance between groups. This repeated measures design also provides for closer comparison of several arms of the experiments and simultaneous assessment of multiple aspects of fluid regulation. Thus we have reorganized the order of experiment execution originally planned, and have focused this first year on examining the water handling ability of the kidneys and the possible changes in vasopressin synthesis and renal receptors as a result of acute and chronic alcohol exposure.

Examination of the ability of the kidneys to excrete a water load:

Acute alcohol effects: In rats administered alcohol acutely for 3 days, the ability to excrete a water load equivalent to 2% body weight was compared to that of control rats. Although time course profiles for water load excretion in both control and acute alcohol exposure groups were similar, with maximal urinary excretion achieved within 30 minutes after

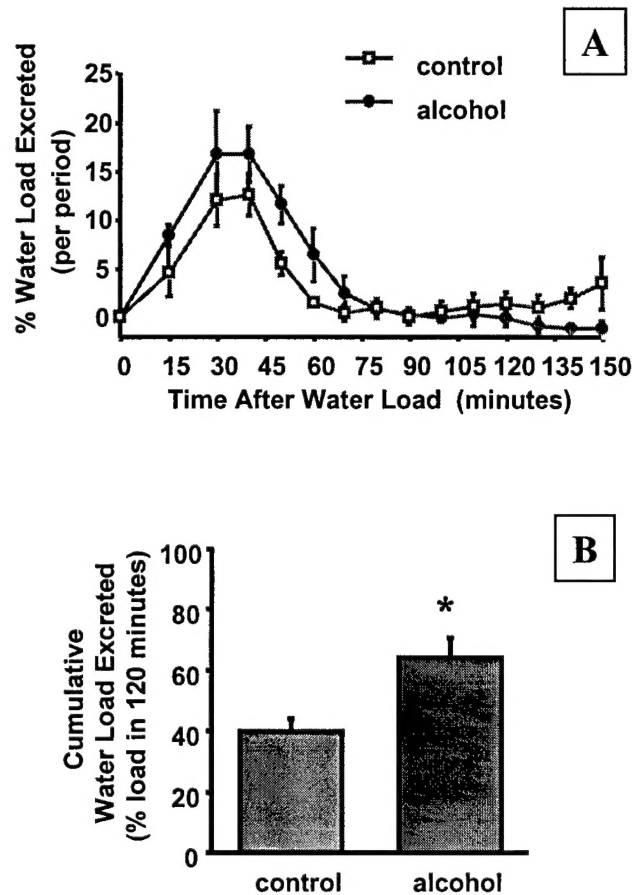


figure 1. Acute alcohol exposure effects on water load excretion ability .

Time course of water load excretion profiles (A) were similar in control group (n=5) and rats 18 hours after acute alcohol exposure (n=5). However, the percent of the total water load excreted after 120 minutes (B) was greater in rats 18 hours after alcohol intake. Values represent means \pm s.e.m. * = significantly different from control $p < 0.05$.

water loading (figure 1A), the total water load excreted within 120 minutes (figure 1B) was greater in rats pretreated with alcohol than in control rats.

This increased water diuresis which was still evident over 18 hours after the last alcohol intake, even after blood alcohol levels were undetectable, could be due to a long-lasting suppression of vasopressin secretion that remains as an after-effect of alcohol exposure, or perhaps an alteration of the renal medullary interstitium tonicity. We will be able to determine if either is the case once vasopressin levels are assessed and V2 agonist experiments testing maximal urine concentrating ability are completed.

Chronic alcohol effects:

As observed in the acute alcohol exposure group, rats with chronic alcohol exposure also exhibited a similar time course of excreting a water load (fig. 2A) as control rats, with maximal water load excretion occurring within 30 minutes after the water load administration in both alcohol and control groups. In contrast to the acute alcohol group, however, the total water load excreted (fig. 2B) was impaired in the chronic alcohol group compared to the control group.

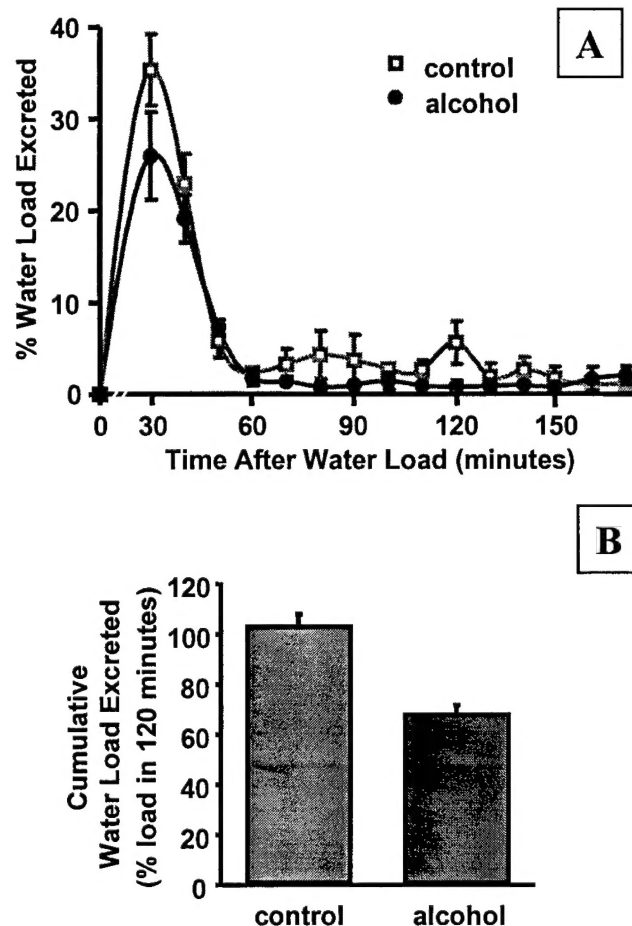


figure 2. Chronic alcohol exposure effects on water load excretion ability.

As observed in rats with acute alcohol exposure, time course of water load excretion profiles (A) in rats exposed to chronic alcohol (n=6) were similar to the excretion profiles in the control group (n=3). In contrast to what was observed with acute alcohol exposure, however, the percent of the total water load excreted after 120 minutes (B) tended to be less in rats exposed to chronic alcohol, indicating an impaired ability to excrete a water load. Values represent means \pm s.e.m.

This impaired ability to excrete a water load is in agreement with that reported in human chronic alcoholics with cirrhosis. However, water impairment in chronic alcoholics with cirrhosis is believed to be caused by the cirrhosis resulting in a splanchnic arterial vasodilation and supposed underfilling of the arterial circulation, causing a nonosmotic release of vasopressin into the circulation and a SIADH-syndrome of water retention (Schrier et al, 1998). In our chronic alcohol model, the rats had no indication of cirrhosis as liver enzyme profiles were within normal range.

We postulate that alcohol may alter renal handling of fluid in chronic alcohol use by affecting regulation of renal V2 receptors involved with tubular water reabsorption. The next series of experiments examining renal dose-response profiles in response to a V2 antagonist support this hypothesis.

V2 antagonist dose response curve generation to examine the renal response to endogenous vasopressin:

Acute alcohol effects: In short term alcohol exposure, the kidneys would not be expected to have yet adapted with a long-lasting change in renal sensitivity in response to acute changes in vasopressin levels. Thus, as might be anticipated, rats in both the acute alcohol and control groups exhibited identical dose-response profiles to V2 antagonist inhibition of endogenous vasopressin (fig. 3). V2 antagonist induced similar changes in urine flow, urine osmolality, free water clearance, and osmotic clearance. Hence, these results indicate that acute alcohol exposure does not alter renal V2 receptor-mediated vasopressin sensitivity.

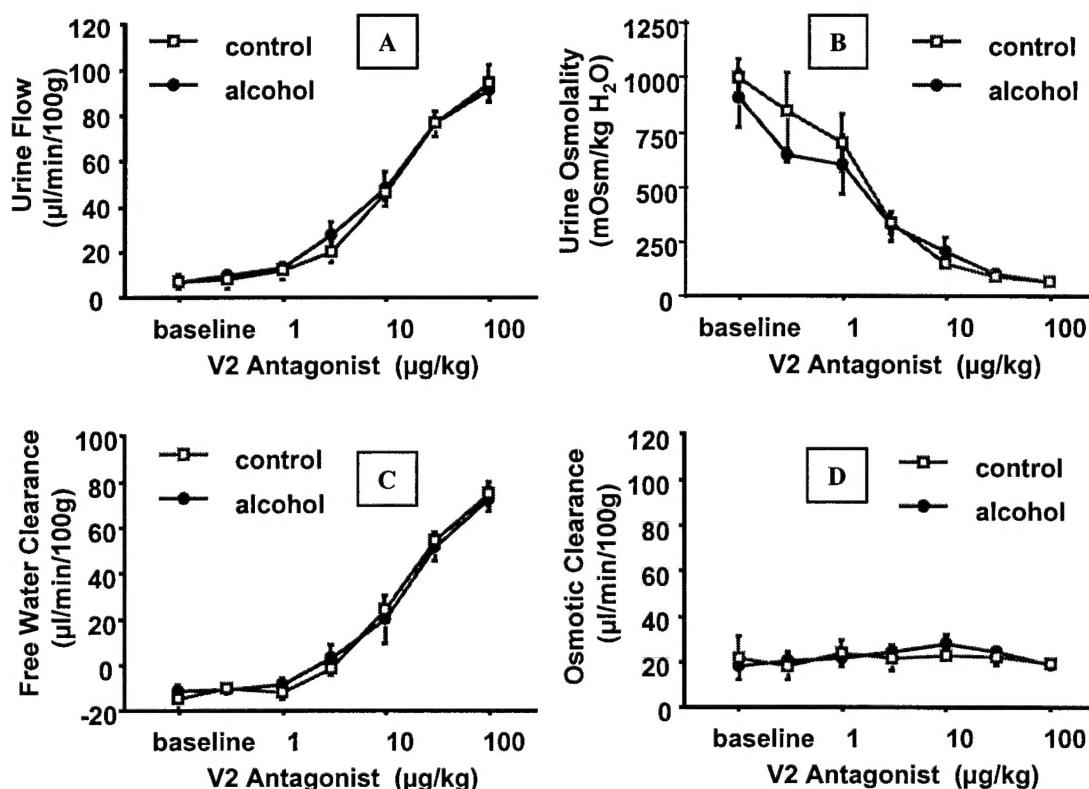


figure 3. Acute alcohol exposure effects on response to V2 Antagonist .

Blockade of endogenous vasopressin with a V2 antagonist induced similar changes in urine flow (A), urine osmolality (B), free water clearance (C) and osmotic clearance (D) in control (n=4) and alcohol-pretreated (n=5) rats. Values represent means \pm s.e.m.

Chronic alcohol effects: In accordance with their impaired ability to excrete a water load, rats chronically exposed to alcohol showed a blunted diuresis (fig. 4) in response to V2 antagonist inhibition of endogenous vasopressin. The suppression of V2 antagonist efficacy in increasing urine flow was due to attenuation of free water clearance in the chronic alcohol group.

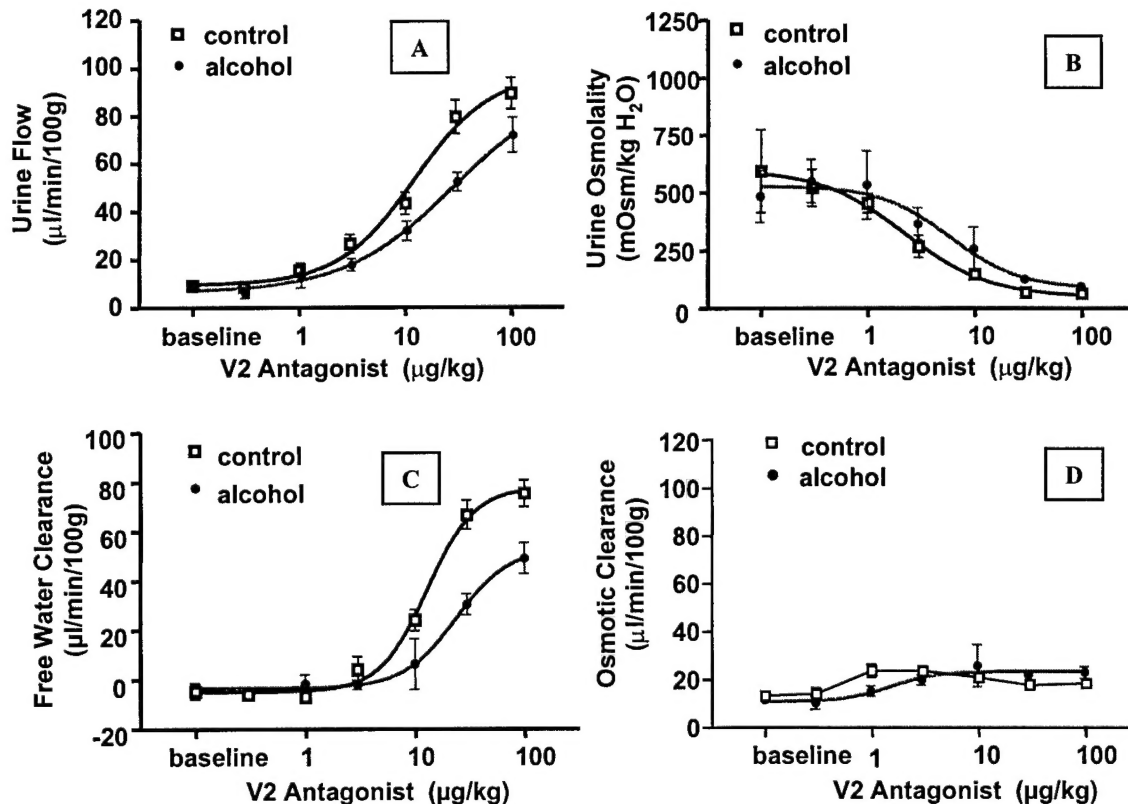


figure 4. Chronic alcohol exposure effects on response to V2 Antagonist.

Rats with chronic exposure to alcohol (n=6) showed a blunted diuresis in response to a V2 antagonist compared to control rats (n=6). Most of the reduced efficacy of V2 blockade on urine flow (A) was predominantly due to a suppressed free water clearance (C) compared to the effect on urine osmolality (B) and osmotic clearance. Values represent means \pm s.e.m.

This decrease in V2 antagonist efficacy occurred despite no apparent differences in plasma vasopressin levels in these rats. Such results are consistent with the hypothesis that impaired ability to excrete a water load and an SIADH-like phenomenon of water retention in chronic alcohol users is due to altered renal responsiveness to endogenous vasopressin. It is possible that an up-regulation of vasopressin receptors in the face of long-term alcohol exposure, similar to that seen with long-term exposure to vasopressin antagonists (Caltabiano and Kinter, 1991) may occur due to lasting changes in steady state circulating levels of vasopressin, or possibly due to direct actions of alcohol itself. Thus, the acute alcohol effect of suppressing vasopressin synthesis and circulating levels results in an up-regulation of vasopressin receptors in the kidney, to compensate for the alcohol-induced diuresis. When eventual compensation by the brain to increase vasopressin synthesis and vasopressin circulating levels occurs in the steady state chronic alcohol exposure phase, the kidney is hypersensitive to vasopressin due to the up-regulation of renal receptors.

At this point, our data from the water load and V2 antagonist experiments are fitting nicely in support of the idea of up-regulation of vasopressin receptors in the kidney. Over the next year, we will be able to definitively characterize this phenomenon with *in vitro* assays of receptor binding and affinity.

V2 agonist dose response curve generation to assess maximum urine concentrating ability with maximal stimulation of vasopressin V2 receptors:

Results of dDAVP dose-response experiments so far suggest that there is no difference in maximal urine concentrating ability between the rats chronically exposed to alcohol (n=6) and control rats (n=6). Because altered water handling in alcohol exposed rats may have indicated an alteration of the renal medullary interstitium tonicity in these animals, the urine concentrating abilities in the face of maximal vasopressin V2 receptor stimulation in these rats were examined. As the results thus far suggest that the rats do not have an altered renal tonicity for fluid reabsorption, it is likely that the altered fluid handling observed is primarily due to V2 receptor density and sensitivity.

Examination of the stimulation of vasopressin release in response to a salt load:

Preliminary results examining the relationship between baseline circulating vasopressin levels and plasma osmolality in the chronic alcohol group so far suggest that this relationship is altered in chronic alcohol exposure. Over the next year we will be able to better define this relationship between vasopressin regulation by osmotic stimulation with the salt loading studies.

Assessment of vasopressin clearance to assess the influence of alcohol on vasopressin metabolism:

We have found interesting results from analysis of vasopressin pituitary and circulating levels (see below) which indicate that vasopressin regulation and clearance may be altered with alcohol exposure. Indeed, if vasopressin receptors are up-regulated in the kidneys as our V2 antagonist results suggest, it is highly likely that renal clearance of vasopressin by the kidneys may be altered.

Alcohol may affect vasopressin receptors similar to the action of vasopressin antagonists which appear to inhibit vasopressin metabolism. It has been shown that administration of either a V1 or V2 antagonist in dogs resulted in an increase in plasma vasopressin (Grove et al., 1998). This is in accordance with the theory that vasopressin renal clearance is receptor mediated (Keeler et al., 1991). If renal vasopressin receptors are somehow affected by chronic alcohol exposure, it is likely that clearance of vasopressin from the circulation will also be affected. We will start experiments assessing vasopressin clearance at the end of year 2.

2. In vitro assessments of tissues and samples to elucidate mechanisms behind altered fluid handling

Measurement of vasopressin levels in the pituitary, blood, and urine:

Chronic alcoholism associated with water retention is supposedly due to increased circulating vasopressin or no change in vasopressin levels but an increase in renal vasopressin sensitivity, impaired renal water excretion, hyponatremia, and cirrhosis of the liver. Alcohol withdrawal, especially in patients with delirium tremens (Trabert et al., 1992) is linked to an increased plasma vasopressin concentration believed to be the result of rebound secretion of vasopressin.

The mechanism behind increased circulating vasopressin levels is not clear because the relationship between vasopressin gene expression in the brain, synthesis, and release has not been systematically studied during the various phases of alcohol exposure and withdrawal. It is possible that vasopressin metabolism by the kidney may be altered, causing circulating levels to increase independent of any change in brain vasopressin mRNA expression and vasopressin synthesis.

We have begun to analyze and compare basal vasopressin levels in acute alcohol, chronic alcohol, and control groups. Preliminary assessment of pituitary vasopressin content and circulating vasopressin levels indicate that the relationship between vasopressin release and circulation is altered with alcohol exposure. In control rats, there is an inverse relationship between plasma vasopressin and pituitary content of vasopressin (fig. 5). This is similar to the

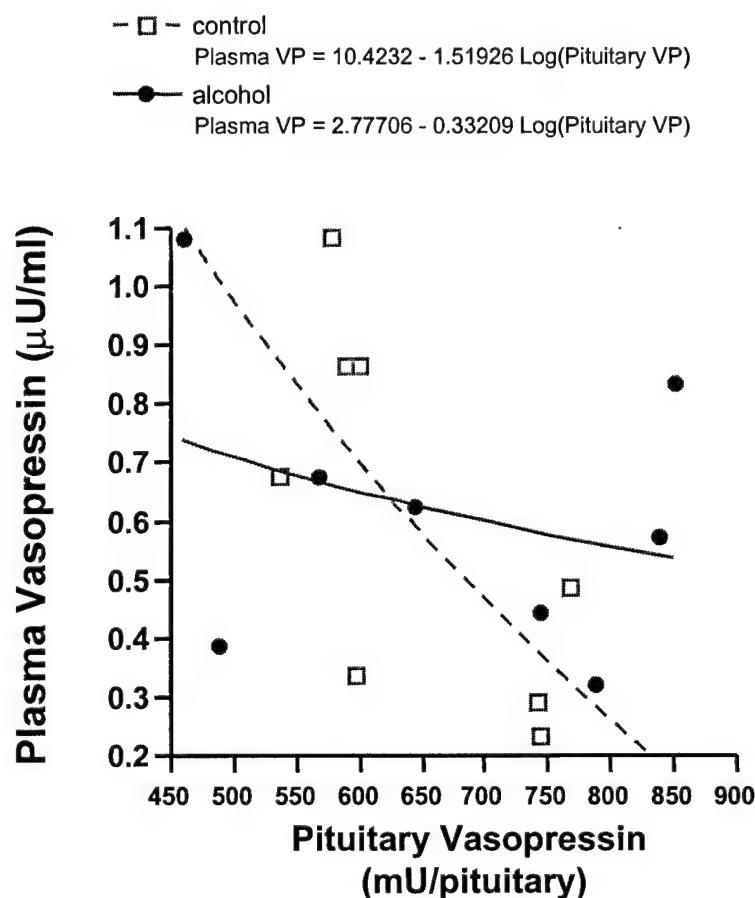


figure 5. Relationship between pituitary vasopressin content and circulating vasopressin.

The inverse relationship ($r = 0.67$) between pituitary vasopressin stores and circulating vasopressin seen in control rats (open symbols, $n = 8$) appears to be disrupted in rats exposed to alcohol (closed symbols, $n = 8$).

relationship previously described in examining the effects of dehydration on vasopressin synthesis and release (Majzoub J.A., 1985). Further, an uncoupling of vasopressin secretion and release into the circulation has been indicated in at least one study where plasma vasopressin levels and plasma osmolality were increased while hypothalamic vasopressin mRNA remained unchanged (Hoffman and Dave, 1991). Another study has also described a disturbance in the rat hypothalamic-pituitary-adrenal axis with just acute alcohol exposure (Lee and Rivier, 1997). Additional assessment of the relationship between vasopressin brain content and blood levels throughout the next 2 years from all rats studied will help us define this apparent uncoupling of vasopressin release and blood levels that occurs with alcohol exposure.

Measurement of brain vasopressin mRNA and vasopressin receptor mRNA:

We have just obtained a real-time quantitative PCR in our laboratory and will be performing the assessment of mRNA levels for both vasopressin and vasopressin receptors over the next 2 years. We delayed starting the mRNA assessments over the first year as our previous methods would generate semi-quantitative comparisons of mRNA levels, but could not discern fine differences. Others have described decreases in vasopressin mRNA and vasopressin neurons as a result of chronic alcohol exposure (Sanna et al, 1993; Gulya et al., 1991; Harding et al, 1996; Ishikawa et al, 1990). With the new technology available in our laboratory, we should be able to provide better quantitation of mRNA levels which can then be used to correlate with circulating and tissue levels of vasopressin and vasopressin receptor protein with greater precision.

Assessment of brain and kidney vasopressin receptor numbers and binding affinity:

Our results from the whole animal experiments conducted this first year clearly indicate that changes in vasopressin receptor numbers or affinity is likely as vasopressin antagonism is not as effective in rats that have been chronically exposed to alcohol when compared to control rats, despite similar circulating plasma vasopressin levels. In year 2 of this project, we will be concentrating on the assessment of the regulation of renal vasopressin receptors.

Assessment of kidney collecting duct cell function:

These experiments to be started in year 2 will help localize the altered renal sensitivity to vasopressin to the collecting duct cells and help define the mechanism of action of the altered renal vasopressin responsiveness in chronic alcohol exposure.

Evidence of alcohol-induced altered regulation of vasopressin synthesis and release.

In addition, we have also begun to characterize hypothalamic content of vasopressin with immunohistochemistry, and early results suggest that brains from rats chronically exposed to alcohol have different density of vasopressin containing cells in the supraoptic and paraventricular nuclei compared to control rats. Over the next year we will continue to examine whether vasopressin synthesis (as indicated by vasopressin mRNA and pituitary content) and release (as indicated by circulating levels) are uncoupled in alcohol exposure. Our immunohistochemistry results will be compared to what is known about vasopressin mRNA expression in response to other conditions such as salt loading (Sherman, 1985) and the brain morphological changes described in other models of chronic alcohol exposure (Maderia, et al, 1993; Ruela et al, 1994).

KEY RESEARCH ACCOMPLISHMENTS:

- Results indicate that even short-term alcohol abuse, equivalent to 3 days of binge drinking, can alter hydration status eighteen hours after the last alcohol drink, as water diuresis appears to persist even after blood alcohol concentrations are back to undetectable levels. This suggests that soldiers need to be adequately rehydrated after any use of alcohol to avoid fluid and electrolyte imbalances that could affect soldier performance in the field.
- Preliminary results suggest that long-term alcohol exposure (equivalent to about 2-3 six packs of beer a day for 8 weeks) affects the ability of the kidneys to process water. This has implications for the effect of alcohol on the regulation of body fluid balance.
- Preliminary results showing chronic alcohol exposure decreases the effect of drug-induced inhibition of the hormone vasopressin on renal water handling suggest that long term exposure to alcohol may cause a compensatory up-regulation of renal receptors for vasopressin, and thus an increased renal sensitivity to vasopressin. This leads to an impaired ability to excrete water and a resultant fluid and electrolyte imbalance.
- Preliminary studies looking at the regulation of brain vasopressin synthesis indicate that alcohol use causes the relationship between brain vasopressin content and circulating vasopressin levels in the blood to be disturbed. This indicates the loss of appropriate linking of the blood levels of this important water regulating hormone with the message the brain receives to synthesize the hormone in response to altered hydration status.

REPORTABLE OUTCOMES

- Animal models for acute and chronic alcohol exposure for precise administration of alcohol that provide a consistent response have been developed for assessment of renal fluid and electrolyte handling. These models also make efficient use of animals enabling reduction of numbers of animals used in research.
- Published abstract and presentation at Experimental Biology 2001:

CFT Uyehara, CA Burghardt, GM Hashiro, and DA Person. After effects of acute alcohol exposure on renal water handling and responsiveness to vasopressin.

FASEB J. 15(4):A134 (Abstract 154.1), 2001 and
J. Investigative Medicine 49(2):249A (Abstract 328), 2001.

CONCLUSIONS:

Despite evidence of impaired renal fluid handling, hyponatremia, and water retention in chronic alcohol exposure and during withdrawal, the renal mechanisms involved and the role of altered vasopressin action in the kidney have not been elucidated. We have shown that the efficacy of endogenous vasopressin on the kidney and resultant changes in renal water excretion is differentially altered in the different phases of alcohol exposure. Rats acutely exposed to alcohol exhibit an increased water diuresis in response to a water load whereas rats chronically exposed to alcohol exhibit an impaired ability to excrete a water load. We have further demonstrated that this impaired ability to excrete a water load in chronic alcohol exposure occurs under conditions of an increased renal sensitivity to vasopressin as demonstrated by suppressed diuretic efficacy of a V2 antagonist. Thus, our results to date have provided evidence of water imbalance with alcohol exposure that is likely due to altered function or numbers of vasopressin receptors, specifically renal V2 receptors, involved with tubular water reabsorption. Additionally, preliminary examination of the relationship between vasopressin pituitary and blood content indicates there is an uncoupling of the regulation of vasopressin release and circulating levels with alcohol exposure.

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